Method for the determination of 25-hydroxycholecalciferol in feed

The present invention relates to a method for the quantitative determination of 25-hydroxycholecalciferol (25-hydroxyvitamin D_3) in animal feed. 25-Hydroxy-cholecalciferol is used as an additive to animal feed and is available as Hy-D™ (ROCHE VITAMINS AG, Basel, Switzerland) to improve the health status of animals such as livestock and pets. In view of its physiological potency and the narrow therapeutic window dosaging of the compound is critical and therefore, reliable analytical means are required to monitor the amount of the compound in feed and its uniform distribution therein. Various methods for the quantitative determination of 25-hydroxycholecalciferol in plasma have been described which are based on immunoassays, see WO 99/67211 or on HPLC/mass spectrometry using derivatives or isotopes as internal standards, see Biological & Pharmaceutical Bulletin (2001), 24(7), 738-743. However, these known methods are not satisfying when applied to the analysis of feed samples.

As all the old methods show it is difficult to analyse 25-hydroxycholecalciferol in feed samples due to the presence of big quantities of solid chemical and biological substances, whereas plasma or serum consist mainly of water. Two types of methods are available. A physico-chemical method using HPLC and UV detection and an immunochemical method using HPLC for sample clean-up and radio-labeled immunoreagents, see Bruce. W. Hollis, Calcif. Tissue Int. (1996) 58:4-5. The other method, is also laborious and contains an analytical step, which uses radioactive material for the quantification. This method consists of the addition of ^{3}H-25-hydroxycholecalciferol as internal standard, extraction with methanol, sample clean-up on reversed-phase SEP-PAK cartridges, further clean-up on normal-phase SEP-PAK cartridges, further clean-up on normal-phase HPLC and final intrinsic analytical reversed-phase HPLC. The overall recovery is determined by scintillation counting of the ^{3}H-25-hydroxycholecalciferol. Quantification is done by external calibration and UV detection at 264 nm.The sample clean-up procedure is so laborious because the final quantification is done by UV. Such a complicated purification of the extract requires a determination of the recovery which is done using radio-labeled

25-hydroxycholecalciferol. Both methods are cumbersome, with many poor performance characteristics and reproducibility.

The present invention provides a novel multistep but straightforward procedure for the quanititative determination of 25-hydroxycholecalciferol which can be applied to animal feed samples with satisfying results.

More particularly the present invention relates to a process for the quantitative determination of 25-hydroxycholecalciferol in animal feed which comprises the steps of

a) dispersing the feed sample in water and adding to the sample a defined amount of an internal standard compound having a mass different from 25-hydroxycholecalciferol and having a polarity similar to but different from 25-hydroxycholecalciferol;

b) extracting the aqueous dispersion with tert.butyl methyl ether;

c) submitting the ether extract to semipreparative HPLC;

d) collecting the fractions containing 25-hydroxycholecalciferol and the internal standard compound;

e) submitting the fractions collected in d) or an aliquot thereof to HPLC combined with mass spectrometry;

f) determining the MS peak areas of 25-hydroxycholecalciferol and of the internal standard compound added; and

g) calculating the amount of 25-hydroxycholecalciferol by computing the MS peak areas measured.

The internal standard compound used in step a) is, e.g., a derivative of, an isomer of or isotopically labeled 25-hydroxycholecalciferol, e.g. a deuterium labeled isotope such as 26,27-hexadeutero-25-hydroxycholecalciferol (Tetrahedron Lett. Vol. 32, No. 24, 2813-2816 (1991); or 25-hydroxyergocalciferol, or 1α-hydroxycholecalciferol. The preferred

standard compound is 26,27-hexadeutero-25-hydroxycholecalciferol. The standard compound is suitably added as solution in methanol prior to dispersion or solution of the feed samplein water. The amount of standard compound to be added to the sample is not narrowly critical. Suitably, the standard compound is added in an amount to provide an about 0.05 m to about equimolar concentration based on 25-hydroxycholecalciferol. The aqueous dispersion or solution of the feed sample is then extracted in step b) with an about 1-10fold mount of tert.butyl methyl ether, preferably with sonication. Semipreparative HPLC in accordance with step c) is accomplished by evaporating the organic solvent from the extract obtained in step b), suitably under exclusion of oxygen, on silica gel using an apolar solvent such as an aliphatic $C_5 - C_8$ hydrocarbon, e.g., isooctane or mixtures of such solvents with other polar solvents, such as lower alkanols, e.g., isopropanol and/or esters, e.g. ethyl acetate. A preferred system for semipreparative HPLC is silica gel and an isopropanol : ethyl acetate : isooctane mixture of about 1 : 10 : 89 (by volume). Analytical HPLC acording to step e) is suitably carried out on a column of an apolar stationary phase such as modified silica gel using a polar solvent such as water or a lower alkanol. The term "modified silica gel" as used herein denotes a reversed-phase silica gel, e.g. silica gel etherified with a C_{18} hydrocarbon moiety, e.g., Aquasil C18 as supplied by Thermo Hypersil-Keystone, Runcorn, UK.

The amount of 25-hydroxycholecalciferol in the sample on the basis of the mass spectrometry measurings according to step g) is calculated by the equations shown below:

$$\mu g\ 25\text{-hydroxycholecalciferol /kg} = \frac{Area_{HD}}{Area_{ISD}} * ng\ ISD * RRF * \frac{1}{Weight[g]}$$

$$RRF = \text{relative response factor} = \left[\frac{RF_{HD}}{RF_{ISD}}\right] = \left[\frac{Area_{HD} * c_{ISD}}{Area_{ISD} * c_{HD}}\right]$$

RF = Response Factor ; RRF = Relative Response Factor ; ISD = Internal Standard Solution; HD = 25-hydroxycholecalciferol; c = concentration [ng/ml].

The relative response factor (RRF) is determined using a solution of both 25-hydroxy-cholecalciferol and 26, 27-hexadeutero-25-hydroxycholecalciferol at approx. 5 ng/ml in a solution of methanol : water (70 : 30).

The invention is illustrated further be the following Example:

Example

A. Extraction: 10 g of a feed sample (comprising a mixture of 28.6 % Soya, 3 % fish meal, 2 % Soya oil, 57.3 % maize, 2 % maize starch, 2 5 lignosulfonate, 3.1 % rice, 2% mineral mix) were weighed into a Erlenmeyer flask. Approx. 500 ng of 26,27-hexadeutero-25-hydroxycholecalciferol (0.01 ml of a solution of 2.5 mg 26,27-hexadeutero-25-hydroxycholecalciferol in 50 ml of methanol) and 60 ml of water were added thereto and the slurry was treated in a sonication bath at 50 °C for 10 min. Then, 40 ml of tert.butyl methyl ether were added, the mixture was vigorously shaken for 5 min. and sonicated again for 5 min and centrifuged. 10 ml of the organic supernatant was separated and evaporated under the exclusion of oxygen.

B. Semipreparative HPLC : The residue was dissolved in 2 ml of mobile phase, isopropanol : ethyl acetate : isooctane (1 : 10 : 89), centrifuged and an 100 µl aliquot from the clear supernatant was injected into a semipreparative HPLC column of Hypersil Si 60, 3 µm, 120 Å, 150 x 4.6 mm, (Shandon). The flow rate was 1.0 ml/min. Fractions between 14-16 minutes were collected (fraction separation was checked by injection of mixed standard solution prior to start) and evaporated in a nitrogen stream at 50 °C. The residue was dissolved in 0.7 ml of methanol using a ultrasonic bath. Then, 0.3 ml of water were added and the solution injected into an analytical HPLC column combined with a mass spectrometer.

C. Analytical HPLC : Analytical HPLC was carried out by means of a chromatography system combined with a mass specific detector. The chromatography system ahead of the mass specific detector consisted of a trapping column, on which the substances to be measured are concentrated, and the intrinsic analytical column for separation.

The installation is schematically depicted in Figure 4. In Fig. 4, "TC" denotes a trapping column, "AC" denotes an analytical column, and "MSD" denotes the mass specific detector. "A" and "B" symbolize receptacles for the mobile phase of the chromatography system in different modes of operation.

In the trapping column (TC) the stationary phase was Aquasil C18, 3 µm, 2.0 x 10 mm

In the analytical column (AC) the stationary phase was Aquasil C18, 3 µm, 3.0 x 150 mm. The mobile phase was water (containing 0.05% HCOOH) and a methanol/water (containing 0.05% HCOOH) gradient. The working parameters of the system were as follows:

Flow rates: Pump 1: 0.6 ml/min
Pump 2: 0.7 ml/min

Injection volume: 90 µl

Injector temp.: 5°C

Column temp.: 40°C

Retention time: approx. 4 min

The chromatography was carried out according to the scheme set forth in Table 1 below:

Table 1

Column Switching System		Trapping Column			Analytical Column		
Time	Position	Time	Mobile Phase [1])		Time	Mobile Phase [1])	
0 – 1.65		0.00	60% B2	Conditioning			
	A	0.00 – 1.00	>85% B2	Loading Concentr.	1 – 1.65	90% B1	Conditioning
		1.00 – 1.65	85% B2	Washing			
1.65 – 2.20	B	1.65 – 2.20	90% B1	**Transfer**, forward flush	1.65 – 2.20	90% B1	Start of chromatography
2.20 – 12.00		2.20 – 2.50	85% B2	Washing	2.20 – 6.40	90% B1	Separation
		2.50 – 2.60	>100% B2	Washing	6.40 – 6.50	>100%	Washing
	A	2.60 – 9.00	100% B2	Washing	6.50 – 9.00	100% B1	Washing
		9.00 – 9.10	>60% B2		9.00 – 9.10	>90% B1	Washing, Conditioning
		9.10 – 12.0	60% B2	Conditioning	9.10 – 12.00	90% B1	Washing, Conditioning

[1]) > = Gradient (change of the composition of the mobile phase)

The parameters of the mass specific detector (MSD) were as follows :

Detector: Agilent 1946C LC/MSD SL single-quadrupole mass specific detector

Ionisation technique: APCI (atmospheric pressure chemical ionisation

Acquisition mode: SIM (selected ion monitoring)

Polarity: positive

Spray and drying gas:	Nitrogen 99.999% (quality N50)
Drying gas flow:	9.5 L/min
Nebulizer gas pressure:	50 psig
Drying gas temperature:	225°C
Vaporizer temperature:	250°C
Capillary voltage:	3000 V (Vcap = ionisation voltage)
Corona current:	10 μA
Gain:	1.5

SIM parameters

Ion	m/z $(M+H)^+$	Fragmentor [V]	Dwell time [msec]	rel. Dwell time [%]
HyD – H_20	383.3	140	226	30
d6-HyD – H_20 ISD	389.3	140	226	30
HyD	401.3	90	151	20
d6-HyD ISD	407.3	90	151	20

Using the above installation and mode of operation, a standard solution, a blank feed sample (no 25-hydroxycholecalciferol present), and a typical feed sample were analyzed. The standard solution was prepared as follows :

1. 25-hydroxycholecalciferol
   2.5 mg of 25-Hydroxy vitamin D_3 were dissolved in 50 ml of methanol. 2 ml of this solution was diluted to 200 ml with methanol to obtain a solution containing 500 ng/ml.
2. d_6-25-hydroxycholecalciferol (internal standard)
   2.5 mg of d_6-25-hydroxycholecalciferol were dissolved in 50 ml of methanol. 2 ml of this solution was diluted to 200 ml with methanol to obtain a solution containing 500 ng/ml.
3. 1 ml each of the solutions of 25-hydroxycholecalciferol (1.) and d_6-25-hydroxycholecalciferol (2.) were diluted to 100 ml with methanol : water (70 : 30) to obtain a solution containing, per ml, 5 ng of the hydroxylated cholecalciferol.

The blank feed sample was analyzed in analogy to the procedure described in paragraph A. above.

The extracted ion chromatograms of the standard solutions, the blank feed sample and the typical feed sample are shown in Figures 1-3. The amounts of 25-hydroxycholecalciferol were calculated by the equations given earlier.